A METHOD FOR ESTIMATION OF THE RELATIVE ANTIGENIC POTENCIES OF PREPARATIONS CONTAINING COMMON NEW ANTIGENS DERIVED FROM A PRECURSOR PROTEIN (β-LACTOGLOBULIN)

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The Schultz–Dale technique has been adapted to the determination of the relative antigenic potencies of the common new antigens in the dialysate fractions (D1–D6), respectively obtained by six successive, 8-min, pepsin hydrolyses of β-lactoglobulin. The dialysate fractions, with a molecular weight of 12,000 or less, amounted to approximately 90% of the starting β-lactoglobulin. Fraction D2, obtained in the highest yield, was used as reference standard in determining the relative antigenic potencies of the other dialysate fractions. Using guinea pigs uniformly sensitized with D2, the relative antigenic potencies were: D1, 0.75; D3, 2.5; D4, 8.0; D5, 3.0; D6, 6.0. Using guinea pigs individually sensitized with the other dialysate fractions, the relative antigenic potencies were : D1, 0.19; D3–D6, 3.0. The precision of the method was ±33%. The new antigens in D1–D6 are essentially as potent as ovalbumin because the minimum amount of new antigen nitrogen which produced a response in the uterine strip of sensitized guinea pig was 0.015 μg as compared with 0.008 to 0.0008 μg of ovalbumin nitrogen.

1. INTRODUCTION

The term 'new antigen' is defined as an antigen with a specificity distinct from that of the protein from which it was generated. This paper describes an adaptation of the Schultz–Dale technique for the estimation of the relative antigenic potencies of the common new antigens generated by pepsin hydrolyses of β-lactoglobulin. Relative antigenic potency (RAP) is defined as the numerical expression of the capacity of a fraction to produce a response in the uterine strip of a sensitized guinea pig relative to that of a reference fraction as determined under standardized conditions. For example, a RAP of 3 indicates that the fraction is three times more potent than the reference fraction.

This method was developed as part of a research on the new antigens generated in a simulated stomach digestion of milk proteins as related to elucidation of the mechanism of milk allergy (Spies et al., 1970). The rationale of the study, details of

the preparations used, and demonstration that each dialysate from six, successive, pepsin hydrolyses contains at least one common new antigen has been described (Spies et al., 1972). The six dialysate fractions, D1–D6, numbered in the order in which they were obtained, amounted to approximately 90% of the original β-lactoglobulin. D2 was obtained in highest yield and hence was used as reference fraction in two series of tests. First, determination of the RAPs of the other fractions using guinea pigs uniformly sensitized with D2. Second, determination of the RAPs of the other fractions using guinea pigs individually sensitized with D1, D3–D6.

The apparatus and conditions for a quantification of the Schultz–Dale technique, as well as examples of its applications in determination of contaminating antigen contents of different proteins have been described by Coulson (1953). The basic principle of Coulson's method was to determine the percentage contraction of a segment of a uterine horn (strip) of a sensitized guinea pig to a measured dose of antigen using the contraction produced by 100 μg of histamine as 100%. The dosages were determined which would give a 20 to 70% contraction to both a reference antigen and to an impure preparation using three to four segments of each strip from a guinea pig sensitized to one of the proteins. The slope of the dosage–response curve was 2.0. The slope was independent of the nature of the antigen used. From these data the amounts of each preparation that would give a 40% response was calculated and the amount of common antigen in the impure protein could be calculated. Coulson observed that different segments of the strip were not uniformly sensitive so he used corresponding portions for reference and test samples.

In our attempts to use Coulson's method for determining the RAPs of the common new antigens in the dialysate fractions, we encountered difficulty in obtaining the required 20 to 70% response with both reference and test fraction on the two halves of the strip because of the narrow dosage range due to the steep slope of the dosage-response curve. We preferred not to cut each strip into three or four pieces because cutting seemed to increase strip irritability, also the magnitude of the response was quite small with these smaller pieces which made precise measurement more difficult. The method described here eliminated or minimized these difficulties and the precision was satisfactory for our purposes.

2. METHODS

2.1. *Pepsin hydrolyses*

The materials and details of the method for preparation of the six fractions of β-lactoglobulin have been described (Spies et al., 1972).

The basic design of these experiments consisted of hydrolysis of β-lactoglobulin with pepsin (2% of the weight of each substrate; C = 50 mg/ml in water) for 8 min at pH 2.0. The reaction was stopped and the hydrolysate was separated into two fractions by dialysis. The dialysates and the endo fractions were isolated by lyo-

Hydrolysis no.	Starting material (g)	Dialysate			
		Symbol of product	Yield [d] (g)	Nitrogen	
				%	% of total [e]
1	42.0 [a]	D1 [c]	4.6	4.1	3.2
2	39.0 [b]	D2	13.6	8.9	23.4
3	23.4 [b]	D3	8.9	8.9	25.6
4	12.4 [b]	D4	4.8	8.4	25.2
5	8.0 [b]	D5	2.7	8.0	20.4
6	4.8 [b]	D6	1.7	8.2	22.3

[a] β-Lactoglobulin.
[b] Retentate from dialysis of preceding hydrolysis.
[c] This D1 was redialyzed yielding 3.5 g of the sample used in this work. Nitrogen content 3.6%.
[d] Inclusive of sodium chloride formed on pepsin hydrolysis.
[e] Based on total nitrogen content of starting sample.

philization. The endo fraction from each hydrolysis was then similarly rehydrolyzed with pepsin; 500 mg of each endo fraction being reserved for immunological analysis. This procedure was done six successive times. Results are summarized in table 1.

2.2. *General Schultz–Dale technique*

Virgin, female guinea pigs, weighing about 225 g, were sensitized by subcutaneous injections (Nuchal area) with two 0.5-ml volumes of dialysate fraction emulsified with an equal volume of Freund's complete adjuvant. The sensitizing dose of dialysate contained 2 mg of dialysate nitrogen. The incubation period was at least 28 days. Challenge doses were administered in terms of total nitrogen in a 50-ml Dale bath. The basic Schultz–Dale technique used, which utilized uterine horns of the sensitized guinea pigs, has been described by Coulson (1953).

2.3. *RAP analysis procedure*

Each excised uterine strip from a sensitized guinea pig was cut into two equal parts. The two ovarian strips were used for one comparison of D2 and test fraction in successive tests using the same bath for both strips. The two vaginal strips were similarly used. This procedure eliminated the effect of differences in the sensitivity of the ovarian and vaginal strips and any individual differences in the two baths. However, in so far as possible, two baths were used simultaneously, one for each pair of strips to cut down storage time, a procedure which lessened their irritability.

Each strip was first challenged with a dosage of a mixture (M) containing equal quantities of nitrogen of each of β-lactoglobulin, pepsin, and PEPD (a previously

Table 2
Guide for calculation of RAP limit relationships of two preparations containing a common antigen.

Response type no.	Antigens [a]	Response [b]	RAP limit $\left(\frac{\mu g\ D2\ nitrogen}{\mu g\ T\ nitrogen} = X\right)$ [c]
1	D2	0	$T > X$
	T	+ to ++++	
2	D2	+ to ++++	$T < X$
	T	0	
3	D2	+ to ++++	$T < X$
	T	+ but < D2	
4	D2	+ but < T	$T > X$
	T	+ to ++++	
5	D2	+	$T = X$
	T	+ equal	
6	D2	0	ND [d]
	T	0	
7	D2	++++	ND [d]
	T	++++	

[a] T = D fraction being compared to reference fraction D2.
[b] Degrees of response of strip: 0 = none; + = submaximal, i.e., < 90%; ++++ = maximal, i.e., 90 to 100%.
[c] X = RAP limit.
[d] Not usable.

described dialysate of a pepsin autodigest (Spies et al., 1972)). Thus, each component of M equaled the quantity of D2 or test fraction nitrogen to be added subsequently. Only strips that gave a negative response to M were used to insure that responses to D2 or test fraction were due to new antigen. After challenging with M, a dosage of D2 or test fraction was added which was based on judgment and experience and the response was noted. The bath was then washed out and after the strip relaxed, a dosage of 100 μg of histamine was added to determine the maximum response of the strip. Positive responses of fractions were measured as per cent of the maximum response. The second segment of the first pair of strips was then similarly challenged with M and then with the fraction being compared. The dosage of this fraction was judged to give a response such that a RAP limit could be determined using the relationships shown in table 2 as a guide. The second pair of strips was similarly tested to give another RAP limit above or below that obtained with the first pair. Thus, for each fraction, two RAP limits were determined, one greater than and one less than the reference fraction D2 within the limits of a two-fold dosage range of D2 and test fraction.

3. RESULTS

3.1. *Dialysates of successive pepsin hydrolysates*

Table 1 contains a summary of the yields, nitrogen contents, and per cent of the total nitrogen of each of the dialysates of the six, successive, pepsin hydrolyses of β-lactoglobulin whose RAPs were determined.

3.2. *Dosage–response relationships in determination of RAP limits*

Table 2 shows the seven types of dosage–response relationships obtainable in determining RAP limits with reference fraction D2. Type 6, where no response was obtained with both fractions, and Type 7, where maximal response was obtained with both fractions, obviously are not usable in RAP limit determinations. The usable types (1–5) of dosage-response relationships are illustrated in fig. 1–5, respectively. The RAP of a fraction was taken as the median value of two RAP limit determinations, one greater than and one less than reference fraction, D2, deter-

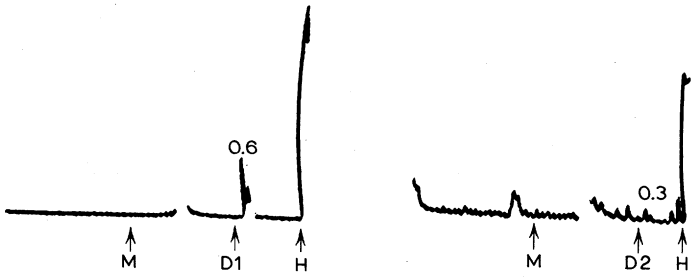

Fig. 1. Example of type 1 (table 2) dosage–response relationships. Sensitizing antigen: D2. Challenge dosages in μg of total fraction nitrogen. RAP limit: D1 > 0.50.

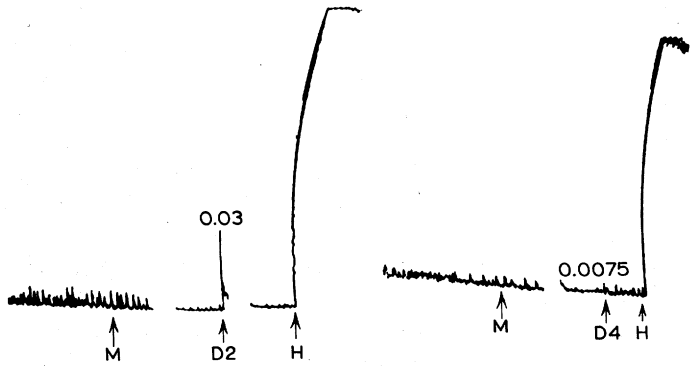

Fig. 2. Example of type 2 (table 2) dosage–response relationships. Sensitizing antigen: D4. Challenge dosages in μg of total fraction nitrogen. RAP limit: D4 < 4.0.

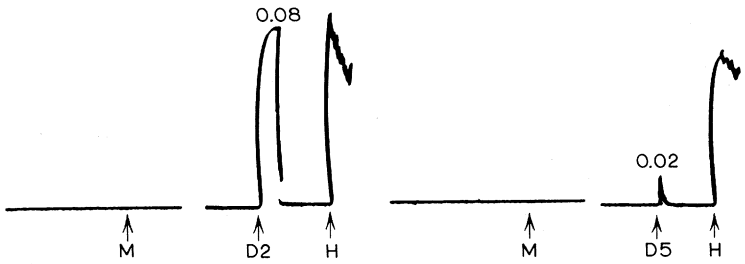

Fig. 3. Example of type 3 (table 2) dosage–response relationships. Sensitizing antigen: D5. Challenge dosages in μg of total fraction nitrogen. RAP limit: D5 < 4.0.

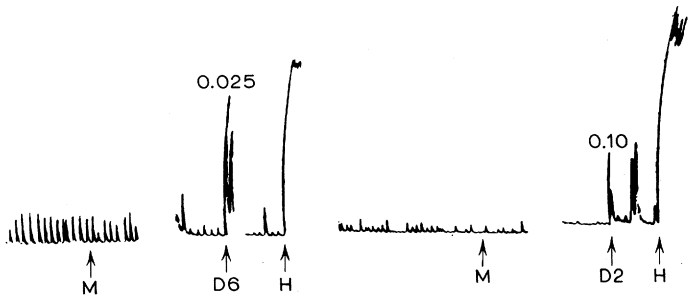

Fig. 4. Example of type 4 (table 2) dosage–response relationships. Sensitizing antigen: D2. Challenge dosages in μg of total fraction nitrogen. RAP limit: D6 > 4.0.

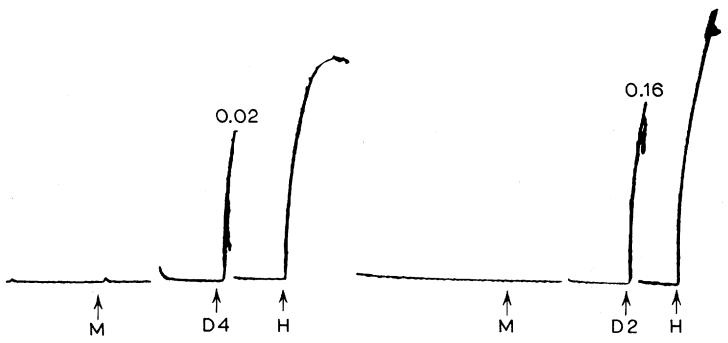

Table 3
RAP limits and RAPs of fractions D1, D3, D4, D5, and D6 using guinea pigs sensitized with D2.

Test antigen		D2 [a] dosage (μg N)	Response type no.	RAP limits	RAP
T	Dosage (μg N)				
D1	0.4	0.1	1	> 0.25	0.75 ± 0.25
	0.6	0.3	1	> 0.50	
	0.4	0.4	3	< 1.0	
D3	0.04	0.16	3	< 4.0	2.5 ± 1.5 [b]
	0.2	0.2	4	> 1.0	
D4	0.02	0.12	1	> 6.0	8.0 ± 2.0
	0.02	0.16	5	8.0	
	0.015	0.15	2	<10.0	
D5	0.02	0.02	4	> 1.0	3.0 ± 1.0
	0.02	0.04	4	> 2.0	
	0.02	0.08	3	< 4.0	
	0.04	0.32	2	< 8.0	
D6	0.025	0.10	4	> 4.0	6.0 ± 2.0
	0.01	0.08	2	< 8.0	

[a] Reference antigen.
[b] These dosage limits were four-fold instead of the usual two-fold because of the unavailability of sensitized guinea pigs. The two-fold dosage limits could have been attained otherwise.

Table 4
RAP limits and RAPs of fractions D1, D3–D6 using guinea pigs sensitized individually with D1, D3–D6.

Test antigen [a]		D2 [b] dosage (μg N)	Response type no.	RAP limits	RAP
T	Dosage (μg N)				
D1	8.0	2.0	3	< 0.25	0.19 ± 0.06
	6.4	0.8	1	> 0.13	
D3	0.05	0.10	1 or 4	> 2.0	3.0 ± 1.0
	0.04	0.16	2	< 4.0	
D4	0.015	0.03	4	> 2.0	3.0 ± 1.0
	0.0075	0.03	2	< 4.0	
D5	0.04	0.04	1	> 1.0	3.0 ± 1.0
	0.03	0.06	4	> 2.0	
	0.04	0.08	4	> 2.0	
	0.02	0.08	3	< 4.0	
D6	0.06	0.06	4	> 1.0	3.0 ± 1.0
	0.05	0.10	1	> 2.0	
	0.025	0.10	2	< 4.0	

[a] Test antigen also sensitizing antigen.
[b] Reference antigen.

mined within the two-fold dosage range. The precision of the RAP determinations was thus ± 33%.

Table 3 shows that the RAPs of fractions D1, D3–D6 were 0.8, 2.5, 8, 3, and 6, respectively, as determined with guinea pigs uniformly sensitized with D2.

Table 4 shows that the RAPs of fractions D1, D3–D6 were 0.2 for D1 and uniformly 3 for all of the other fractions, as determined with guinea pigs sensitized individually with D1, D3–D6.

Challenges on strips from at least 3 non-sensitized guinea pigs were all negative with 10 μg of nitrogen of each of D1–D6 and of M.

3.3. *Minimum dosages of dialysate nitrogen producing response*

Table 5 contains a summary of the minimum dosages of fraction nitrogen observed which produced a specific response in sensitized strips. The minimum dosage with strips from guinea pigs uniformly sensitized with D2, exclusive of D1, ranged

Table 5
Minimum observed dosages of D1–D6 fractions producing a response in strips from guinea pigs sensitized with D2 and with D1, D3–D6.

Sensitizing fraction	Challenge		Sensitizing fraction	Challenge	
	Fraction	Minimum dosage (μg N)		Fraction	Minimum dosage (μg N)
D2	D1	0.4	D1	D1	6.4
D2	D2	0.02	D1	D2	1.5
D2	D3	0.04	D3	D3	0.05
D2	D4	0.02	D3	D2	0.10
D2	D5	0.02	D4	D4	0.015
D2	D6	0.02	D4	D2	0.030
			D5	D5	0.02
			D5	D2	0.08
			D6	D6	0.05
			D6	D2	0.10

from 0.02 to 0.04 μg of D2–D6 nitrogen. The minimum dosage with strips from guinea pigs sensitized separately with fractions D2–D6 was from 0.015 to 0.05 μg nitrogen for the sensitizing fraction and slightly higher from 0.03 to 0.1 for D2 nitrogen. The minimum dosages of D1 for D2 and D1 sensitized strips was another order of magnitude higher at 0.5 μg nitrogen for a D2 sensitized strip and 6.4 μg for D1 nitrogen and 1.5 μg of D2 nitrogen for a D1 sensitized strip.

4. DISCUSSION

Three important relationships or properties of the D1–D6 series of fractions were determined by use of the method described: (1) determination of the RAPs of the fractions; (2) estimation of the threshold quantities of nitrogen of each fraction required to produce a response in strips of sensitized guinea pigs; and (3) confirmation that D1–D6 contained common antigens with a specificity distinct from that of precursor β-lactoglobulin. The method has general applicability for other similar series of preparations. Also the RAPs of fractions with respect to the antigenic specificity of the precursor protein could be determined using guinea pigs sensitized with the original protein.

A significant observation was that there was a considerable increase in potencies of the nitrogen of fractions from successive pepsin hydrolyses. Thus from data in table 3, using guinea pigs uniformly sensitized with D2, it is apparent that D2 is 0.75 times more potent than D1 in producing a response, but fractions D3–D6 varied from 2.5 to 8 times more potent than D2. From table 4, using guinea pigs individually sensitized with D1, and D3–D6, it is apparent that D2 is 5 times more potent than D1 in producing a response and that D3–D6 are uniformly 3 times more potent than D2. Thus, as β-lactoglobulin is progressively hydrolyzed by pepsin, fractions with a molecular weight of 12,000 or less, containing common new antigens of increased potency, continue to be split off up to 90% splitting of the β-lactoglobulin.

The new antigens in fractions D3–D6 are almost as potent as ovalbumin, a known, extremely potent antigen, in their capacities to produce a response in strips of sensitized guinea pigs. According to Coulson (1953), a highly sensitive strip responded to 0.0008 μg of ovalbumin nitrogen while the majority responded to 0.008 μg or less. In this study the lowest dosage of new antigen fraction nitrogen observed to produce a response was 0.015 μg of D4 nitrogen, a quantity only twice as much as that which produced a response with the majority of strips with ovalbumin and only 18 times that which produced a response with the most sensitive strips with ovalbumin.

Possible explanations for the increased RAPs of D3–D6 are: presumed lower molecular weights of components which carry the antigenic determinants common to D2 or lower content of non-antigenic components in D3–D6 than that of D2. Further study will be required to determine the accuracy of these speculations.

The significance of these findings in elucidation of the mechanism of the allergic response to ingested milk proteins, and undoubtedly other food proteins, is discussed in more detail in the paper describing the isolation and characterization of fractions D1–D6 (Spies et al., 1972).

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